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The Possible Rôle of Gut Bacteria in Nutrition and Growth of the Sea Hare *Aplysia*

by

TIMOTHY Z. VITALIS, MARGOT J. SPENCE,
AND THOMAS H. CAREFOOT

Department of Zoology, University of British Columbia, Vancouver, Canada V6T 2A9

Abstract. The rôle of bacteria in nutrition and growth of the sea hare *Aplysia* was investigated by: (1) comparing growth of *Aplysia juliana* Quoy & Gaimard on three algal diets in seawater media with and without antibiotics, (2) assessing the number and types of bacteria in the guts of *Aplysia dactylomela* Rang and *A. juliana* on different algal diets, (3) determining in vivo and in vitro effects of various antibiotics on these bacteria, (4) measuring the degree to which bacteria isolated from the gut of *A. juliana* break down various algal storage products and structural polysaccharides, and increase the pool of free amino acids in a culture medium containing the green alga *Ulva fasciata* Delile, and (5) assessing possible direct effects of antibiotics on the sea hare itself.

Aplysia juliana grew significantly less under antibiotic treatment (10 mg penicillin-G and 10 mg streptomycin sulfate per L seawater) on each diet. The antibiotic treatment reduced the number of gut bacteria in *A. juliana* by approximately one order of magnitude. Twelve colony types (assumed to represent 12 bacterial types) were found in the gut of *A. juliana* and 23 different types in *A. dactylomela.* Five bacterial types seemed to be residential in the two species and varied little with changes in diet. Bacteria isolated from the gut of *A. juliana* were capable of breaking down carbohydrates in test media and increasing the pool of free amino acids in a culture medium of the green alga *Ulva fasciata.* Neither behavior nor rate of oxygen consumption in *Aplysia* was significantly affected by exposure to concentrations of antibiotics up to five times those employed in the growth studies, suggesting that the antibiotics reduced the nutritional contribution of the bacteria by reducing their numbers in the gut, rather than having a directly deleterious effect on the sea hares themselves. The results suggest that gut bacteria may contribute significantly to nutrition and growth in *Aplysia.*

INTRODUCTION

The relationship between animals and their symbiotic gut bacteria is well understood in vertebrates and in commercially and medically important insects (BROOKS, 1964; MCBEE, 1971). In marine invertebrates, however, the relationship is not as clear. Several recent studies on marine invertebrate herbivores have pointed to a nutritional rôle for the gut bacteria (PRIM & LAWRENCE, 1975; FONG & MANN, 1980; CAREFOOT, 1981a, b), although the extent of nutritional contribution in some forms has been questioned (LAWRENCE, 1975; PRIM & LAWRENCE, 1975). FONG & MANN (1980) showed clearly that amino acids produced by gut bacteria in the sea urchin *Strongylocentrotus droebachiensis* were absorbed from the gut and used in the synthesis of the host's own tissues. FISHER & CHILDRESS (1986) also demonstrated that a large proportion (>45%) of the carbon fixed by symbiotic chemautotrophic bacteria inhabiting the gills of the gutless bivalve *Solemya reidi* is translocated to, and used by, the host. In neither study was the importance of such contribution to the host animal's overall nutrition investigated. Indirect evidence that the sea hare *Aplysia kurodai* may employ bacterially produced amino acids in its own nutrition was provided by CAREFOOT (1981b). There, young animals maintained steady weight on several chemically defined artificial diets with deficiencies in amino acids known to be essential for the rat, over a 24-day experimental period. The most likely explanation for this was that gut bacteria were providing the necessary amino acid nutrients, assuming that sea hare requirements for amino acids are the same as found for other animals. Another study on juvenile *A. dactylomela* (CAREFOOT, 1981a) showed that animals eating a chemical diet deficient in arginine (essential for the rat) and containing antibiotics gradually lost weight over a 20-day period. The weight loss was reversed when the arginine-deficient diet was

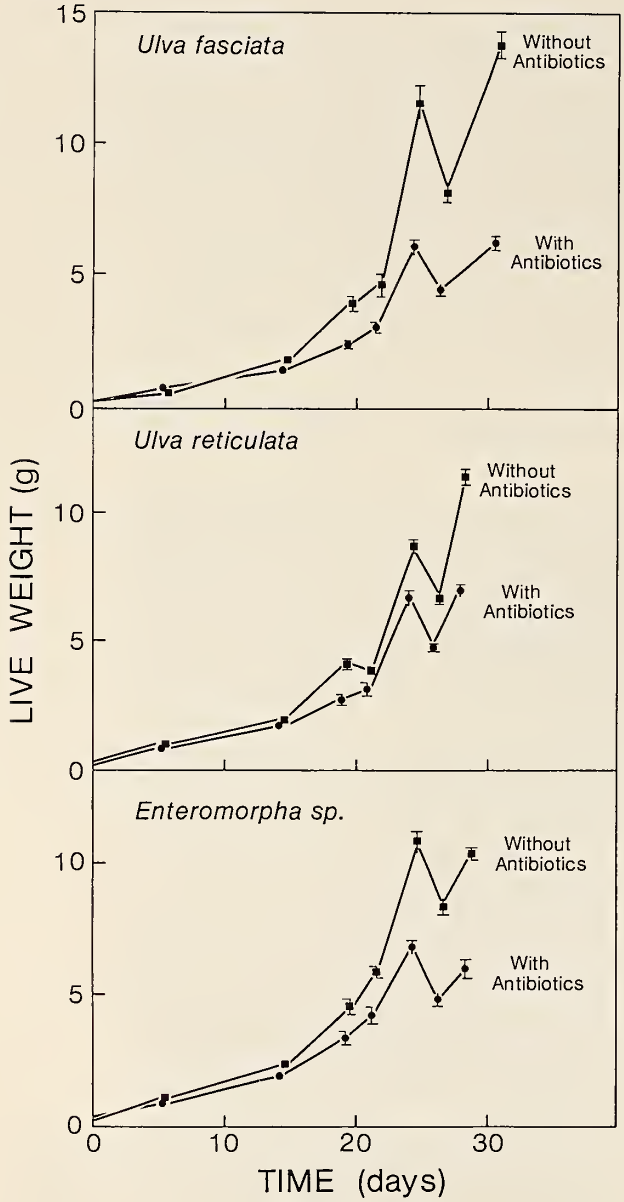

Figure 1

Change in live weight of *Aplysia juliana* on diets of the green algae *Ulva fasciata, U. reticulata,* and *Enteromorpha* sp., with and without antibiotic treatment (10 mg streptomycin sulfate and 10 mg penicillin-G·L seawater^{-1}) at 25°C. Each point represents the $\bar{X}$ ± SE of six animals.

replaced by one complete in all nutrients. In vitro studies by Prim & Lawrence (1975), on the ability of bacteria isolated from the guts of sea urchins to digest algae and various storage products of algae, have further supported the notion that symbiotic gut bacteria are generally involved in the nutrition of marine invertebrate herbivores. This contribution could be especially significant in instances where less than optimally nutritious seaweeds or other plants are eaten, where foods are particularly indigestible, or where for reasons of temporal or spatial shortages a variety of seaweeds must be consumed.

The present study investigates the possible rôle of gut bacteria in the nutrition and growth of the sea hare *Aplysia.* It involves five major approaches: (1) a comparison of growth rates of animals treated and not treated with antibiotics, (2) an assessment of the number and types of bacteria in the guts of the sea hares, and whether these change with change in diet, (3) an assessment of the effects of various antibiotics on these bacteria, (4) a measure of the degree to which algal storage products and structural polysaccharides are broken down by bacteria isolated from the guts of sea hares, and the extent to which bacteria can increase the pool of free amino acids in an *Ulva fasciata* culture medium, and (5) an assessment of the direct inhibitory effects, if any, that the antibiotics might have on the sea hares themselves.

Effect of Antibiotic Treatment on Growth Rates

Individuals of *Aplysia juliana* were metamorphosed from a single batch of eggs following procedures outlined in Switzer-Dunlap & Hadfield (1977). Forty-two animals were successfully metamorphosed. When they reached a live weight of 0.15–0.30 g, 36 were divided into 6 groups of 6 animals each, such that each group had approximately the same mean live weight and variance (0.25 ± 0.001 g). Each group was kept in a plastic tub (10 × 10 × 14 cm) and supplied with its own flow of seawater (33‰, 25°C). The groups were randomly divided into three double sets (control and experimental), each set receiving as food one of the green seaweeds *Enteromorpha* sp., *Ulva fasciata,* or *U. reticulata* Forsskal. After a 5-day holding period, the experimental group in each set was treated with antibiotics in an attempt to sterilize the animals' digestive tracts. The antibiotic treatment consisted of 10 mg streptomycin sulfate and 10 mg penicillin-G delivered per L of seawater over the 28-day period of study. The antibiotics were administered dropwise from a stock solution (containing 1 g of mixed antibiotics per L seawater) into the seawater flowing through the system. At the start of the antibiotic treatment all animals were given rations just sufficient to satisfy their appetites (based on data from previous growth studies; Carefoot, 1970). This minimized "superfluous" feeding (feeding in excess, which results in quicker passage of food through the gut and less efficient digestion) and thus ensured that the gut bacteria had maximal time to act on the ingested algae. The animals were weighed every few days over the 28-day experimental period.

Figure 1 shows change in live weight of *Aplysia juliana* on three seaweed diets with and without antibiotics. In each case, animals treated with antibiotics grew significantly less than did untreated animals ($P < 0.001$, ANOVA). Animals grew fastest and showed greatest response to the antibiotics on a diet of *Ulva fasciata,* the alga most

Table 1

Description of bacterial colonies isolated from the guts of *Aplysia juliana* (Nos. 1–6) and *A. dactylomela* (Nos. 1–4, 6–24). Standard descriptive characteristics from BUCHANAN & GIBBONS (1974).

	Macroscopic colony morphology							Microscopic colony morphology				
No.	Size (mm)	Shape	Margin	Pigmentation	Colony under reflected light	Colony under transmitted light	Elevation	Gram stain	Shape	Spore-forming	Arrangement	
1	2–3	round	entire	creamy	smooth	opaque	slight	–	cocci	no	pairs random	20% 80%
2	0.5–1.5	round	entire	white	smooth	opaque	slight	–	cocci	no	pairs triplets	70% 30%
3	5–6	round	entire	yellow-creamy	rough	transparent	slight	–	rods: long and slender	no	random	100%
4	0.5–1	round	entire	yellow	smooth	opaque	raised	–	cocci	no	pairs chains random	20% 40% 40%
5	2	irregular	entire	orange, grainy	smooth	transparent	very slight	–				
6	1–3	round	entire	orange	smooth	opaque	slight	–	rods: length/width = 2/1	no	chains random	50% 50%
7	varying	irregular	convoluted	yellow	rough	transparent	slight	–	cocci	no	random	100%
8	0.5–1	round	entire	yellow	smooth glossy	opaque	very slight	–	cocci	no	random	100%
9	varying	irregular	convoluted	pale yellow	smooth	transparent	very slight	–	rods	no	random	100%
10	0.5–1	round	entire	colorless	smooth	transparent	raised	–	cocci	no	random	100%
11	10	irregular	convoluted	creamy edge, dark center	smooth	transparent	slight	–	cocci	no	pairs random	20% 80%
12	0.5–1	round	entire	creamy	smooth	opaque	slight					
13	1–2	round	convoluted	colorless	smooth	transparent	slight	–	rods	no	random	100%
14	1	round	entire	black	smooth	opaque	slight	–	cocci	no	random	100%
15	2–3	round	entire	cream with dark center	glossy smooth	opaque	raised	–	rods: length/width = 2/1	no	random	100%
16	1–1.5	round	entire	colorless	smooth	transparent	slight	–	rods: length/width = 3/1	yes	random	100%
17	varying	irregular	convoluted	yellow	rough	opaque	slight	–	cocci	no	random	100%
18	2	round	irregular	yellow	rough	opaque	very raised	+	cocci	no	random	100%
19	varying	irregular	irregular	colorless	smooth	transparent	slight					
20	≤0.5	round	entire	colorless	smooth	transparent	none					
21	1–4	round	entire	white	smooth	opaque	slight	–	rods: length/width = 2/1	no	pairs random	20% 80%
22	2	round	entire	pink center, white edge	smooth	opaque	very raised					
23	1–3	round	entire	cream	smooth	transparent	slight	–	rods: length/width = 3/1	no	grouped random	40% 60%
24	0.5–1	round	entire	colorless	smooth	transparent	slight	–	cocci	no	chains random	45% 55%

Table 2

Number and types of bacteria isolated from the guts of *Aplysia*. Values are based on the means of counts from 2 to 6 animals.

Diet	Total number of colony types	Predominant colony types		Number of bacteria/mL gut fluid
		Number	Identity (Nos. refer to Table 1)	
Aplysia juliana				
Ulva spp.	12	6	Nos. 1–6	43×10^5
Enteromorpha sp.	12	6	Nos. 1–6	—
Aplysia dactylomela				
Ulva fasciata	7	4	Nos 1, 2, 4, 9	53×10^5
Spiridea sp.	17	5	Nos. 1, 7, 9, 10, 24	—
Laurencia sp.	10	7	Nos. 1, 4, 7–9, 19, 24	—
starved (5 days)	11	4	Nos. 1, 4, 9, 24	—
artificial	—	—		25×10^5

preferred by field animals and one known to be a good settlement-inducer in this species of sea hare (SWITZER-DUNLAP & HADFIELD, 1977). Overall, the antibiotic treatment reduced growth of the animals by 40–53% on the three diets. Neither diet nor the interaction of antibiotics and diet had a significant effect on growth rates ($P = 0.11$ in each case, ANOVA). A pump malfunction in the seawater system bathed the animals for one day with supersaturated seawater (a leak caused air to be pumped into the seawater under pressure), and this feeding interruption explains the conspicuous drop in weight shown by all animals on day 26 of the experiment (see Figure 1).

Number and Types of Bacteria in the Guts of *Aplysia*

At the end of the growth experiment the gut was dissected from each *Aplysia juliana* and the total contents of the crop and gizzard regions removed with a sterile Pasteur pipette. The contents were diluted 1000-fold in autoclaved seawater and an aliquot plated onto nutrient agar. The culture medium consisted of 10 g bactopeptone (Difco), 15 g agar (Difco), and 1 L of membrane-filtered and autoclaved seawater. To this was added 15 mL 50% w/v separately autoclaved glucose solution. Two culture media were used, one with a pH of 6 to match that of the crop of *Aplysia,* the other with a pH of 8 to match that of the gizzard. The inoculated plates were incubated for 4 days at 25°C, after which the colonies were counted and characterized according to standard morphological features (methods outlined in BUCHANAN & GIBBONS, 1974). The bacteria were not identified as to species. Similar platings were made from the guts of *A. dactylomela* feeding on several algal diets, from some that had been starved for 5 days, and from ones eating a chemically defined artificial diet.

Twenty-four colony types were identified in platings from crop and gizzard contents of *Aplysia juliana* and *A. dactylomela* (Table 1). In *A. juliana,* six of these isolates were abundant and appeared consistently (Nos. 1–6, Table 1; another six appeared in the *A. juliana* platings, but only sporadically and in low numbers, and thus are not described in Table 1). There was no obvious correlation between the presence or absence of a bacterial type and the diet or treatment (with or without antibiotics) in this sea hare species. The number of bacteria was highly variable, but averaged about $43 \times 10^5 \cdot \text{mL}$ gut fluid^{-1} in normal animals, and $4\text{–}10 \times 10^5 \cdot \text{mL}$ gut fluid^{-1} in antibiotic-treated animals.

In comparison, 23 bacterial types were identified in platings from the buccal mass, crop, gizzard, and digestive gland of *Aplysia dactylomela.* Of these, 4–7 types predominated depending on diet, 4 of which were present in the guts of animals starved for 5 days. There was no evident correlation of bacteria types with seaweed diet in *A. dactylomela* (Table 2). Animals starved for 5 days had 11 types spread through the various regions of the gut, as many or more than in the guts of some normally feeding animals. Of the 6 commonest types isolated from *A. juliana,* 5 appeared identical to types from *A. dactylomela* (Nos. 1–4, 6; Table 1). All but one of the total of 24 different types present in abundance in the two species of *Aplysia* had Gram-negative staining characteristics. Finally, there was no consistent pattern either in the types or numbers of bacteria with the location in the gut in *A. dactylomela.*

Effect of Antibiotics on the Gut Bacteria

The effectiveness of antibiotics in reducing the numbers of gut bacteria in *Aplysia juliana* was determined by treating six animals (11–14 g live wt) over a 6-day period with seawater containing antibiotics (50:50 mix of streptomycin sulfate and penicillin-G) in concentrations ranging from 0 to 1000 mg·L seawater^{-1}. All animals were allowed to feed *ad libitum* on *Ulva fasciata* and *U. reticulata.* The animal in zero concentration of antibiotics was a control. At the end of the 6-day period of treatment the gut contents were removed and plated onto agar discs, as described

Table 3

Resistance of selected bacterial types from the gut of *Aplysia dactylomela* (colony Nos. 10 and 24, Table 1) to various antibiotics. The antibiotics were administered in the form of Difco-discs containing standardized antibiotics dosages. Response of the bacteria is indicated as S = susceptible, I = intermediate susceptibility, and R = resistant.

Antibiotic	Dosage in each disc (μg)	Response of bacteria	
		No. 10	No. 24
Ampicillin	10	I	I–S
Chloromycetin	10	S	S
Erythromycin	5	I–S	R
Kanamycin	10	I	R
Neomycin	10	I–S	R
Penicillin	3	R	R
Polymycin B	10	I	R
Streptomycin	5	R	R
Tetracycline	10	R	I–S

previously, and the total number of bacteria growing at each dosage of antibiotics was counted.

Figure 2 shows the bacterial cell counts in relation to antibiotic dosage. The antibiotics appeared to reduce markedly the number of bacteria, even at comparatively low dosages. The control *Aplysia juliana* had 43 × 10^5 bacteria·mL^{-1} in its crop/gizzard, comparable to values of 48 and 59 × 10^5 bacteria·mL^{-1} counted in the digestive contents of two specimens of *A. dactylomela* feeding on *U. fasciata* (see also Table 2). The *A. juliana* treated with 70 and 1000 mg antibiotics·L seawater^{-1} each had counts of 1 × 10^5 bacteria·mL gut fluid^{-1}. Based on these observations and bacterial counts of animals used in the growth experiments, the concentration of antibiotics used in the growth experiment reduced the number of gut bacteria approximately one order of magnitude.

A number of antibiotics were tested in vitro for their effectiveness against gut bacteria in *Aplysia dactylomela.* Difco discs containing standard dosages of the antibiotics ampicillin, chloromycetin, erythromycin, kanamycin, neomycin, penicillin, polymycin B, streptomycin, and tetracycline were tested against two of the most prevalent types of bacteria found throughout the guts of sea hares (Nos. 10 and 24; see Table 1). The standard dosages in the discs were 10 μg for all except erythromycin and streptomycin, which were 5 μg, and penicillin which was 3 μg. The response of *Escherichia coli* to the same antibiotics was used as a standard of comparison for the responses of the *Aplysia* gut bacteria. A standard test procedure was employed as outlined in Buchanan & Gibbons (1974).

The results of the Difco-disc series of experiments are presented in Table 3. Ampicillin and chloromycetin were the most effective antibiotics on the two bacterial types tested. With the exception of some intermediate levels of susceptibility of one or other of the bacterial isolates to erythromycin, neomycin, and tetracycline, the bacteria were generally resistant to all of the other antibiotics.

Utilization of Plant Constituents by *Aplysia* Gut Bacteria

Utilization of various plant constituents by bacteria isolated from the gut of *Aplysia juliana* was determined using carbohydrate-vitamin-nitrogen (CVN) media as described by Prim & Lawrence (1975). Each CVN medium contained a single carbohydrate source. Carbohydrates tested included glucose, maltose, starch, carrageenan, and cellulose. After a 4-day incubation at 25°C with each of the six main bacterial types isolated from *A. juliana,* utilization of a carbohydrate was judged to have occurred if the culture medium turned cloudy, if sediment accrued at the bottom, or if growth occurred at the surface (Prim & Lawrence, 1975). CVN media without bacteria and inoculated CVN media without a carbon source were used as controls. Each

Table 4

Utilization of carbohydrates by each of six types of bacteria isolated from the gut of *Aplysia juliana.* The bacteria were incubated for 4 days at 25°C in carbohydrate-vitamin-nitrogen media at pH = 6 (as found in the crop of *Aplysia*) and pH = 8 (gizzard).

Bacterial isolate	Carbohydrate substrate									
	Glucose		Maltose		Starch		Carrageenan		Cellulose	
	pH 6	pH 8	pH 6	pH 8	pH 6	pH 8	pH 6	pH 8	pH 6	pH 8
1	+	+	+	+	−	+	+	+	−	−
2	+	+	−	−	−	−	+	+	−	−
3	+	+	+	+	+	+	+	+	−	−
4	+	+	+	+	−	−	+	+	−	−
5	+	+	+	+	+	+	+	+	−	−
6	+	+	+	+	+	+	+	+	−	−
Control (no carbohydrate)	−	−	−	−	−	−	−	−	−	−
Control (no bacteria)	−	−	−	−	−	−	−	−	−	−

Table 5

Concentration of free amino acids in 10-mL aliquots of three *Ulva fasciata* media: one, incubated 4 days at 25°C with a mixture of the six predominant bacterial types from the gut of *Aplysia juliana*; the other, a sterile control medium incubated 4 days at 25°C; the last, a fresh homogenate of the seaweed. The final column gives the approximate ×-fold change in concentration of each amino acid resulting from bacterial action. Dashes indicate no data available. Amino acids in block type are those known to be essential for the rat.

	Concentration (μM) of free amino acids in *Ulva fasciata* media			
	Control (incubated 4 days with no bacteria)	Fresh homogenate	Incubated 4 days with gut bacteria	×-fold change in amino acid concentration
ARGININE	—	trace	516	—
Alanine	17	62	319	+8
Aspartic acid	22	34	32	nil
Glutamic acid	23	14	214	+11
Glycine	25	59	661	+16
HISTIDINE	trace	41	23	nil
ISOLEUCINE	10	12	—	—
LEUCINE	16	16	—	—
LYSINE	16	20	21	nil
METHIONINE	7	—	134	+19
Ornithine	26	38	24	nil
PHENYLALANINE	10	7	107	+12
Proline	—	—	461	—
Serine	34	110	50	nil
Threonine	11	22	12	nil
TYROSINE	9	7	49	+6
VALINE	—	173	114	nil

test of a carbohydrate was replicated three times at pH values of 6 and 8, representing values recorded from the crop and gizzard, respectively, of *A. juliana*.

Table 4 shows that all six bacterial isolates were able to grow on the CVN media containing either glucose or carrageenan at pH levels present in the crop and gizzard. The maltose- and starch-containing media showed lack of growth in some instances. Neither the cellulose-containing medium nor the controls supported growth of any of the bacteria.

In addition, the ability of the gut bacteria to digest seaweed was tested; a change in the concentration of free amino acids in the culture was used as a measure of bacterial activity. Two 50-mg samples of oven-dried (60°C) *Ulva fasciata* were each mixed with separate 2.5-mL portions of seawater to slurry consistency and autoclaved for 15 min. Following this, one sample (the control) was made up to 10 mL with 3.75% sulfosalicylic acid in 0.2 N lithium citrate buffer, sealed, and stored in the dark at 2°C. The remaining sample was made up to 10 mL with autoclaved seawater and inoculated with a mixture of the six predominant bacterial isolates from *Aplysia juliana* and incubated for 4 days at 25°C. At the end of the 4-day period, a third 50-mg sample of dried *Ulva* was homogenized in 0.2 N lithium citrate buffer and made up to 10 mL. All three samples were then centrifuged at 13,000 RPM for 5 min at 0°C. Aliquots (100 μL) of the supernatant were analyzed for the presence of free amino acids using a Beckman Model 118C amino acid analyzer.

Table 5 indicates that bacterial action markedly increased the pool of free amino acids in a culture medium containing only seawater and the alga *Ulva fasciata*. The values for the test culture were compared with the averages of values for control (no bacteria) and freshly prepared homogenate. There was no consistent difference between these last two media with respect to levels of free amino acids, suggesting that the bacteriocidal sulfosalicylic acid treatment had no, or only minor, chemically degrading effect on any free amino acids originally present in the homogenate. Several essential (for the rat) amino acids showed marked increases in concentration following incubation of the seaweed with the bacteria (methionine, phenylalanine, tryosine, and possibly arginine), as did some non-essential ones (alanine, glutamic acid, and glycine). Several amino acids remained in much the same concentration as at the start, while none showed any marked decrease in concentration.

Other Effects of the Antibiotics

The question remains as to whether antibiotic treatment had any effect on the sea hares beyond reducing their levels of gut bacteria. The animals' feeding behavior was not inhibited by antibiotic treatment. None of the animals died during the experiments and none showed evidence of poor health. However, since this was a critical point in interpretation of the results, a further experiment was performed to show that metabolic rate was not markedly affected by antibiotic treatment. *Aplysia dactylomela* was used in this experiment instead of *A. juliana,* as none of the latter was available. The two species are similar in size and share common behavioral attributes (*e.g.,* both are nocturnal). *Aplysia dactylomela* readily eats the ulvoid species favored as food by *A. juliana* and growth rates are comparable in the two species. The experiment employed an antibiotic concentration five times that in the *A. juliana* growth study (a total of 100 mg antibiotics·L seawater^{-1}), and tested the immediate effects of the antibiotics on oxygen uptake. A flow-through respirometer system with a YSI Model 58 Dissolved-Oxygen Meter was used to monitor oxygen levels (see Carefoot, in press, for details of respirometry methods). The antibiotics were administered directly from a stock solution into the seawater line flowing to the respirometer. An animal was allowed 30 min of equilibration time in the respirometer before its rate of oxygen uptake was measured. Then, to test for instantaneous effects of the antibiotics, a 1-h baseline level of oxygen uptake was established before the supply of normal seawater was switched to that containing antibiotics. Rates of oxygen uptake were monitored for a further 2-h period after the start of antibiotics administration. All measurements were made between 1100 and 1400 h at a temperature of 28.5°C during the animals' normal daytime state of quiescence.

Figure 3 shows that there were no apparent acute effects of the antibiotics in oxygen consumption in *Aplysia dactylomela.* Rates were maintained at a fairly constant level of 3.75–3.85 mL $O_2 \cdot indiv^{-1} \cdot h^{-1}$ ($P = 1.00$, ANOVA) despite perfusion of antibiotics-containing seawater (five times the concentration used in the growth experiment) over the *Aplysia.* Because it took some time for the antibiotics-treated seawater to replace completely the normal seawater in the respirometer flask, and because this varied with each run (depending on volume of the flask and flow rate), the data in Figure 3 were re-expressed to show the rate of oxygen consumption in relation to the actual concentration of antibiotics. These data are presented in Figure 4 and again show no significant effect of the antibiotics on the rate of oxygen uptake in *Aplysia* over short-term exposure ($P = 0.99$, ANOVA). While a larger sample size and further information on possible long-term effects of the antibiotics on the metabolic rate in *Aplysia* would make these results more convincing, the data do suggest that the level of antibiotics employed in the growth study had no directly deleterious effects on the sea hares.

DISCUSSION

Several points of interest emerge from these data. First, antibiotic treatment significantly reduces the growth of juvenile *Aplysia juliana.* Second, bacteria are abundant both in the number of individuals and the types throughout the gut of sea hares, including the buccal region, crop, gizzard, and digestive gland. Finally, bacterial isolates from the gut of *A. juliana* readily break down various plant constituents, such as maltose, starch, and carrageenan, and increase the concentration of free amino acids in a seaweed culture medium.

These facts point to a possible nutritional contribution by the gut bacteria to their hosts. However, an alternative explanation is that the antibiotics directly inhibit growth of juvenile sea hares. Of the two antibiotics used in the growth study, penicillin is the least likely to produce growth-inhibiting side-effects in metazoans. Its antibacterial activity involves the disruption of formation of cross-linkages in the structurally unique bacterial cell wall (Franklin & Snow, 1975) and its action appears to be specific against bacteria (Hammond & Lambert, 1978). In comparison, streptomycin affects bacteria more generally, inhibiting protein synthesis on ribosomes by interfering with the coding sequence of amino acids in the elongating peptide chains (Hammond & Lambert, 1978). Streptomycin is known to inhibit specifically the incorporation into peptide linkages of arginine, glutamic acid, histidine, phenylalanine, and threonine (Franklin & Snow, 1975). The potency of a penicillin and streptomycin mixture results from synergistic effects of the component antibiotics, which reduce bacterial cell counts more effectively than if either antibiotic is used individually (Brown *et al.,* 1979). The blocking of cross-linking in the peptidoglycan structure of the bacterial cell wall by penicillin facilitates the movement of streptomycin into the cell (Brown *et al.,* 1979). Of the two antibiotics, then, streptomycin would appear to be potentially most deleterious to the sea hares.

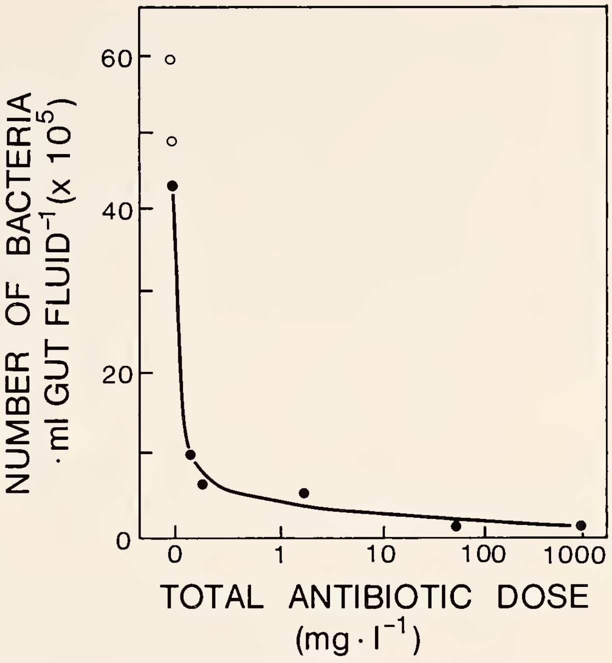

Figure 2

Change in number of bacteria in the gut contents of *Aplysia juliana* after a 6-day exposure to different concentrations of antibiotics in the seawater bathing the animals, at 25°C. The antibiotic solution was a 50:50 mixture of streptomycin sulfate and penicillin-G in seawater. Solid dots, *A. juliana*; open dots, *A. dactylomela.* The latter were normal (untreated with antibiotics) animals feeding for the same length of time as *A. juliana* on the same green alga foods, *Ulva fasciata* and *U. reticulata,* and are included for comparison. Each point represents a single animal.

Chernin (1959) tested the effects of various antibiotics on the freshwater snail *Australorbis glabratus* under axenic conditions. He found that streptomycin stunted growth of the snail at concentrations of 100 $\mu g \cdot mL^{-1}$. No inhibition occurred at a concentration of 10 $\mu g \cdot mL^{-1}$ (equivalent to that used in the present study), and the author noted that small additions of $CaCl_2$ to the culture medium negated certain stress-induced behavioral effects caused by the antibiotic, even at fairly high concentrations (100 $\mu g \cdot mL^{-1}$; Chernin, 1959). Penicillin had no effect on growth of *Australorbis* at concentrations up to 60 $\mu g \cdot mL^{-1}$ (Chernin & Schork, 1959; six times the concentration used in the present study). Thus, if sea hares responded in a manner

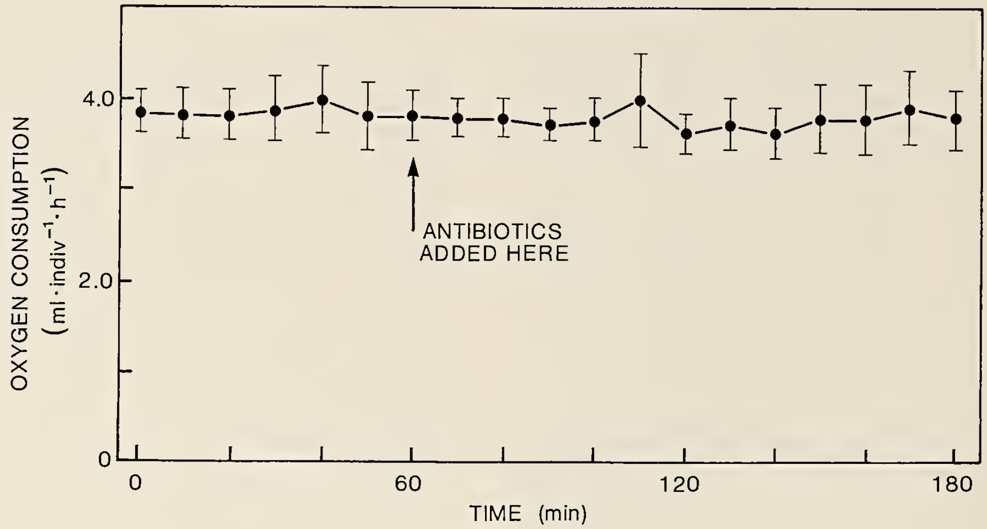

Figure 3

Effect of antibiotics on oxygen uptake in *Aplysia dactylomela.* After 60 min to establish a baseline level of oxygen consumption, seawater containing 100 mg antibiotics$\cdot L^{-1}$ (50 mg each of streptomycin sulfate and penicillin-G) was introduced into the respirometer at normal rates of flow. Owing to a scarcity of animals only four experiments were run. Animal weights ranged from 150 to 307 g live. All values for oxygen consumption (V_{O_2}) were converted to a standard animal weight of 200 g live using the equation $V_{O_2(200\,g)} = 200/\text{exper wt})^{0.926} \cdot V_{O_2(\text{exper})}$ (see Carefoot, 1987). Each point represents the $\bar{X}$ ± SE. Temperature = 28.5°C.

similar to *Australorbis,* the decreased growth rate of *Aplysia juliana* could not be attributed to an antibiotic effect. As well, the relatively high concentration of calcium ions in seawater could have ameliorated any potentially undesirable behavioral side-effects streptomycin might have had on the sea hares.

Oxygen consumption measurements on *Aplysia dactylomela* treated with five times the concentration of antibiotics employed in the *A. juliana* growth study showed no significant deviations from baseline values. If the antibiotic treatment had created stress or caused illness in the sea hares it would presumably have been manifested as a change in metabolic rate, reflected in either increased or decreased levels of oxygen consumption. As noted earlier, the animals appeared to be normal throughout the growth experiment and, even when subjected to a 50-fold greater concentration of antibiotics than that used in the growth experiment (Figure 2), the sea hares showed no observable change in activity or in the rate of feeding. While these observations are circumstantial, they nonetheless support the idea that the negative effects of the antibiotics on the growth of *A. juliana* were indirect effects due to a reduction in the numbers of gut bacteria, and not directly deleterious effects on the sea hares themselves.

Bacteria certainly seem to be involved in the breakdown of plant materials in the guts of marine invertebrate herbivores. They have been shown capable of releasing glucose from carbohydrate storage materials such as maltose and starch, and from such structural materials as carrageenan (but not cellulose) during in vitro studies using bacteria from sea urchins (Prim & Lawrence, 1975) and bacteria from sea hares (present study, see Table 4). In addition, bacterial isolates from the gut of *Aplysia juliana* have been shown in the present study to increase the concentration of free amino acids in an *Ulva fasciata* culture medium. The absorption and utilization of such bacterially produced amino acids and fixed carbon by host animals in the synthesis of their own tissues have been demonstrated in the sea urchin *Strongylocentrotus droebachiensis* (Fong & Mann, 1980) and the gutless bivalve *Solemya reidi* (Fisher & Childress, 1986). While no similar experiments have been done with sea hares, it can be expected that any bacterially mediated breakdown product of seaweeds, whether glucose or amino acids, would be readily absorbed and used by an animal in its own metabolism. Indeed, there could even be a degree of exploitative competition for food resources occurring within the gut between the bacteria and host.

The sea hare has several possible sources of amino acids due to bacterial activity: (1) from normal extracellular digestion of algal protein by the bacteria, (2) from synthesis by bacteria and the release to the gut lumen, (3) from digestion of bacteria by other bacteria, (4) from autolysis of bacteria, and (5) from digestion of bacteria by the sea hare's own digestive enzymes. The high steady-state concentration of gut bacteria (even in starved animals), combined with their diversity, point to a potentially important rôle played by them in the nutrition of their hosts. Fong & Mann (1980) suggest, in this regard, that the ability of sea urchins to use a wide variety of seaweeds as foods

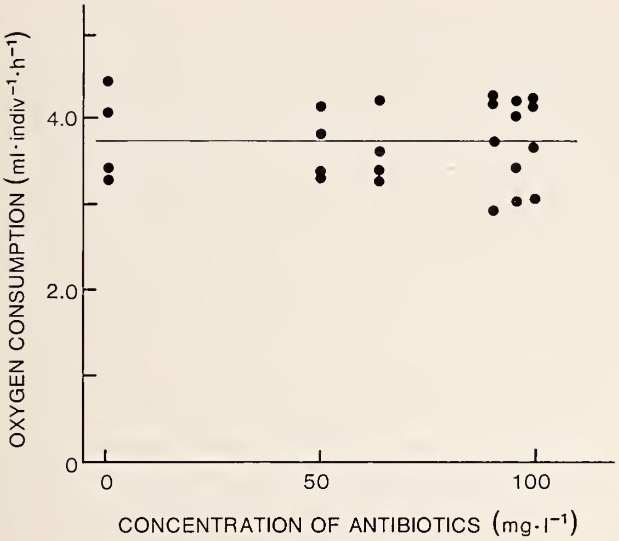

Figure 4

Relationship of oxygen consumption to antibiotics concentration in *Aplysia dactylomela.* Data from the experiment outlined in Figure 3. Regression statistics for the equation $Y = a + bX$ are $a = 3.74$ and $b = -0.093$. The correlation coefficient $r = -0.138$ is not significantly different from zero ($P > 0.50$).

stems from the clandestine nutritional contributions made by bacterial symbionts.

In summary, antibiotic treatment inhibited growth of juvenile sea hares either through an unknown and unobserved directly deleterious effect or through an indirect effect of nutrient loss through reduction in the number of gut bacteria. The post-metamorphic rate of growth is high in sea hares, and thus demand for amino acids is high. Removal of gut bacteria, a potential source of amino acids and glucose beyond those available from the breakdown of algal protein, would limit the rate of protein synthesis and metabolism and thus limit growth. We showed an approximately one order of magnitude reduction in the number of gut bacteria in *Aplysia* after treatment with antibiotics. This is considerably less than the five orders of magnitude reduction shown by Fong & Mann (1980) to occur in the sea urchin *Strongylocentrotus droebachiensis* after 2 days of treatment with the same antibiotics (but using a higher concentration: 90 mg·L seawater^{-1}) but, nevertheless, represents a considerable reduction in bacterial numbers. We believe that gut bacteria do contribute significantly to the nutrition of *Aplysia,* as they seem to do for other marine invertebrate herbivores, and represent a factor largely neglected in past studies of growth, nutrition, and dietary preferences in marine invertebrate herbivores in general.

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